

Single point mutations located outside the inter-monomer domains abolish trimerization of *Schizosaccharomyces pombe* PCNA

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Received February 23, 1998; Revised and Accepted April 15, 1998

ABSTRACT

We have generated proliferating cell nuclear antigen (PCNA) mutants by low fidelity PCR and screened for lethal mutations by testing for lack of complementation of a *Schizosaccharomyces pombe* strain disrupted for the *pcn1⁺* gene. We thus identified eight lethal mutants out of the 50 cDNAs tested. Six were truncated in their C-terminal region due to the introduction of a stop codon within their coding sequences. Two were full-length with a single point mutation at amino acid 68 or 69. The two latter mutants were overexpressed in insect cells via a recombinant baculovirus and were purified. They were unable to stimulate DNA polymerase δ DNA replication activity on a poly(dA)-oligo(dT) template. Cross-linking experiments showed that this was due to their inability to form trimers. Since these two mutations are adjacent and not located in a domain of the protein putatively involved in inter-monomer interactions, our results show that the β -sheet β F1 to which they belong must play an essential role in maintaining the 3-dimensional structure of *S.pombe* PCNA.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a nuclear protein which was primarily identified as a processivity factor of DNA polymerase δ (pol δ) (1–3). The formation of a PCNA-containing replication complex at the replication fork has been extensively studied (4–7) and can be summarized as follows. PCNA is charged onto DNA by the five subunit complex replication factor C (RF-C) in an ATP-dependent manner, thus forming a sliding clamp. After sliding along the double-strand DNA, the RF-C-PCNA complex becomes anchored at the primer-template junction. This complex tethers the polymerase onto the template and a processive holoenzyme is formed. The interaction of PCNA with FEN-1 (8), a nuclease involved in the maturation of Okazaki fragments, suggests that PCNA is involved in all steps of DNA replication and constitutes a moving platform that anchors factors involved in DNA replication to DNA.

Recently, besides its role in DNA replication, PCNA has been implicated in nucleotide excision repair (9) and base excision repair (10). A direct interaction between PCNA and MSH2–MSH3 heterodimers also suggests that it plays a role in DNA mismatch repair (11,12). In addition, PCNA might be involved in cell cycle control. In mammalian cells, PCNA is found in large complexes containing a cyclin, a cyclin-dependent kinase and p21 (13). It has been shown that PCNA can interact directly with D-type cyclins (14). The direct interaction between PCNA and p21 has been extensively studied and results show that p21 can specifically inhibit DNA replication but not DNA repair (15). In addition, overexpression of PCNA in *Schizosaccharomyces pombe* cells results in a G2 delay (16). So, PCNA may constitute a link between a DNA damage checkpoint and cell cycle arrest (for a review see 17).

The gene encoding PCNA has been cloned in many organisms, from yeast to human (for a review see 18). It is an essential gene in *Saccharomyces cerevisiae* (19) and *S.pombe* (16). The crystal structure of *S.cerevisiae* PCNA has been resolved (20). It shows that three PCNA molecules, each containing two topologically identical domains, are tightly associated to form a closed circular ring. The central cavity is positively charged and large enough to allow the passage of a double-stranded DNA molecule. The outside of the ring is negatively charged and provides a platform for the attachment of polymerases and other partners of PCNA. The resolution of the crystal structure of human PCNA associated with a p21 peptide showed a remarkable structural similarity with *S.cerevisiae* PCNA (21). Despite the almost total absence of sequence homology, the eukaryotic PCNA structure closely resembles that of the β subunit of *Escherichia coli* DNA polymerase III (22), which also functions as a sliding clamp.

As PCNA appears to be multifunctional, specific regions of the protein are probably involved in interaction with other proteins implicated in DNA replication, DNA repair and cell cycle control. Site-directed mutagenesis has been performed on human, *S.pombe* and *S.cerevisiae* PCNA, either on charged amino acids or on amino acids conserved across evolution (23–27). Random mutagenesis has also been carried out on *S.cerevisiae* PCNA (28). In yeast, this mutagenesis yielded a wide range of growth defects and sensitivity to DNA damaging agents, certain of which could be traced to anomalous interaction with specific proteins of the replication machinery. No thermosensitive mutations were isolated

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and only cold-sensitive mutants have been described. A cold-sensitive mutant which had a mutation localized at the subunit interface was unable to trimerize *in vitro* (25). Other cold-sensitive mutations (28) are clustered in the interdomain region of the PCNA monomer structure and are defective for DNA replication. On the other hand, amino acids essential for interaction of *S.pombe* PCNA with proteins of the replication machinery could still support cell growth (26). Altogether, site-directed mutagenesis emphasized the fact that PCNA was remarkably resistant to mutations insofar as many of them were silent mutations.

In order to identify residues essential for PCNA function *in vivo*, we designed a screen to isolate lethal mutations. We carried out mutagenesis of the *S.pombe pcn1*⁺ gene by low fidelity PCR, which introduced one or two mutations per molecule. By random spore analysis, we tested the ability of 50 independent PCR molecules to complement a *S.pombe* strain deleted for the *pcn1*⁺ gene. By this method we identified eight mutants that were unable to complement the disrupted strain. Two of them were single point mutations and were further characterized *in vitro*. Our results show that a single point mutation, which lies outside the monomer interacting domain, can affect the 3-dimensional structure of PCNA.

MATERIALS AND METHODS

Cells and strains

The genotype of the *S.pombe* strain used for mutant screening is *h*⁺/*h*⁺ *ura4D18/ura4D18 leu1-32/leu1-32 ade6-M210/ade6-M216 pcn1*⁺/*pcn1*⁺::*ura4*⁺. It was constructed by transforming the parental strain SP826 with a linear fragment carrying the *ura4*⁺ gene surrounded by 5' and 3' sequences of the *pcn1*⁺ gene. The linear fragment was extracted from plasmid pPCNA::Ura4, which was constructed as follows. A *Bam*HI–*Acc*I *pcn1*⁺ fragment [from the *Bam*HI site upstream of the ATG (29) to the first internal *Acc*I site, 380 bp downstream of the ATG] was blunted by the Klenow fragment of DNA polymerase I and cloned into the blunted *Pst*I site of plasmid pUra4-c (a pUC119 plasmid with the *ura4*⁺ gene cloned into the *Sph*I site; from P.Russel, Scripps Clinic). A *Xho*I–*Bam*HI *pcn1*⁺ fragment [from the unique internal *Xho*I site to the *Bam*HI cloning site (29)] was blunted and cloned into the *Kpn*I blunted site of pUra4⁺. Both *pcn1*⁺ fragments were cloned in reverse orientation relatively to *ura4*⁺ sequences. The *Pst*I (110 bp downstream of the *pcn1*⁺ ATG)–*Nhe*I (50 bp downstream of the stop codon) fragment was extracted from pPCNA::Ura4 and transformed as a linear fragment into strain SP826. A strain deleted of one allele of the *pcn1*⁺ gene was selected and characterized by Southern blot.

Insect cells, *Spodoptera frugiperda* (Sf9), were maintained either as a monolayer culture or in spinner bottles as previously described (29).

PCR mutagenesis

Single point mutations in the *pcn1*⁺ gene were obtained by modification of standard PCR conditions (31). PCR was performed on PCNA cDNAs, obtained by reverse transcription, in a final volume of 50 µl buffer A (200 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.005% Tween-20, 0.005% NP-40) containing 400 µM each dNTP, 100 pmol each primer *pcna*-5' and *pcna*-3' as previously described (29), 3 mM MgCl₂ and 2.5 U thermostable DNA polymerase (Replitherm).

Plasmids constructs

The PCNA cDNAs generated by PCR were 1084 bp long and had a *Bam*HI site at each end. These fragments were cloned into the *Bam*HI site of plasmid pEPT, which is a pART1-based vector (32) where the *adh* promoter has been replaced by the weak *polI*⁺ gene promoter. This plasmid was constructed as described (32) except that the *Sph*I–*Pst*I fragment containing the *adh* promoter was replaced by a 420 bp *Pst*I–*Dra*I fragment containing the entire intergenic region between *polI*⁺ and *Rpl7b* (33), which was cloned at *Pst*I–*Hinc*II of pUC18.

For overexpression in Sf9 cells, mutant PCNA cDNA were cloned into the *Bam*HI site of the baculoviral transfer vector pBacPak1 (Clontech). Recombinant baculoviruses were obtained as previously described (29).

Yeast transformation

Schizosaccharomyces pombe transformation was performed essentially as previously described (30). Cells were grown in YE + 2% glucose until they reached a concentration of 5 × 10⁶ to 2 × 10⁷ cells/ml. The cells were harvested, washed once in LTE (0.1 M LiCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA), resuspended in LTE at a concentration of 5 × 10⁸ cells/ml and incubated for 30 min at 30°C. Aliquots of 150 µl of these competent cells were mixed with 1 µg DNA and 350 µl PEG 50% (50% PEG 4000, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA). After 30 min incubation at 30°C, cells were heat shocked for 15 min at 42°C. After removal of the PEG, cells were plated onto selective medium and placed at 30°C.

Random spore analysis

After transformation with the plasmids carrying potential mutant PCNA cDNA, *S.pombe* transformants were selected on EMM. A *h*⁹⁰ clone was identified by iodine staining after plating 10 000 cells on EMM. Sporulating colonies were patched on EMM and grown for 2 days. A loopful of the patch was inoculated in sterile water containing 1000 U β-glucuronidase (Sigma), for digestion of asci. After overnight incubation at 30°C, 500 spores were plated on each selective medium.

Sequencing

Wild-type and mutant PCNA cDNA were cloned into the *Bam*HI site of plasmid pGEM3 for sequencing. The primers used were either universal primers [T7 (Ozyme) and SP6 (Promega)] or internal primers defined on the basis of the *pcn1*⁺ sequence. Sequences were performed with the T7 sequencing kit (Pharmacia Biotech), according to the supplier's recommendations.

Protein extracts and western blotting

Schizosaccharomyces pombe protein extracts were prepared from 1 × 10⁸ mid log phase cells grown in selective medium. Cells were washed with distilled water and broken with 1 ml glass beads by vortexing four times for 1 min. Beads were washed with 800 µl extraction buffer (20% ethylene glycol, 10 mM Tris–HCl, pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT and 1 µg/ml each leupeptin, pepstatin and aprotinin). The extract was centrifuged for 15 min at 12 000 g and the supernatant was recovered. Soluble proteins were separated by 12% SDS–PAGE, transferred onto nitrocellulose and detected with a 1/3000 dilution of the PCNA monoclonal antibody PC10 (Boehringer).

PCNA purification

Wild-type or mutant PCNA proteins were purified from 3×10^8 insect cells infected with the corresponding recombinant baculovirus, essentially as previously described (1). Cells were broken by sonicating six times for 30 s at 1 min intervals in buffer B (40 mM Tris-HCl, pH 7.5, 100 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT). After centrifugation at 27 000 *g* for 20 min, the supernatant was applied to a 2 ml DEAE-cellulose column equilibrated with buffer B. After washing with the same buffer, proteins were eluted with 500 mM KCl in buffer B. Fractions containing PCNA were pooled, dialyzed against buffer TGEED (50 mM Tris-HCl, pH 7.8, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 50 mM KCl) and incubated for 2 h with 1.5 ml phosphocellulose pre-equilibrated in buffer TGEED. The phosphocellulose flow-through was dialyzed against buffer KGD (20 mM KPO₄, pH 7, 20% glycerol, 1 mM DTT) and applied to a 1 ml hydroxyapatite column pre-equilibrated in the same buffer. After washing the column with 500 mM KCl in buffer KGD, proteins were eluted with a linear gradient of KPO₄ (20–300 mM) in buffer KGD.

Protein cross-linking

Cross-linking experiments with ethyleneglycol bis(succinimidyl succinate) (EGS) were performed as previously described (34). The reaction mixtures contained 30 µg/ml wild-type or mutant PCNA. The products were separated by 8.5% SDS-PAGE, transferred onto nitrocellulose and detected by western blot using monoclonal PCNA antibodies PC10.

DNA polymerase assays

The standard reaction mixture of 25 µl contained 50 mM bisTris, pH 6.5, 1 mM DTT, 250 µg/ml BSA, 6 mM MgCl₂, 10 µM [³H]dTTP (1846 c.p.m./pmol), 500 ng poly(dA)₃₀₀-oligo(dT)_{12–18} (ratio 10:1) and 0.35 U *S.pombe* pol δ. Wild-type or mutant PCNA was added as indicated in the figure legend. DNA synthesis was carried out at 37°C for 30 min. The reaction was stopped by addition of 10% (w/v) cold trichloroacetic acid. The insoluble material was adsorbed onto glass microfiber filters and counted in a Beckman scintillation counter (LS3801). One unit of pol δ catalyzes the incorporation of 1 pmol nucleotide in 60 min at 37°C.

RESULTS

Isolation of random lethal mutants of PCNA

Our strategy to identify lethal mutants of PCNA is summarized in Figure 1. In *S.pombe*, PCNA is encoded by the *pcn1*⁺ gene. We used the fact that *pcn1*⁺ is an essential gene to identify mutant PCNA unable to complement, after sporulation, a *S.pombe* strain disrupted for the *pcn1*⁺ gene. Single point mutations were introduced at random by low fidelity PCR on cDNA encoding PCNA. PCR-amplified cDNAs were cloned into the *Bam*HI site of plasmid pEPT and isolated clones were checked for correct orientation. The pEPT plasmid is a pART1-based (32) plasmid where the strong *adh* promoter has been replaced by the weak *S.pombe polI*⁺ gene promoter. Indeed, PCNA, when overexpressed in *S.pombe* under either the strong *adh* or *nmt1* promoters, displays a toxic effect (16). We checked that expression of wild-type PCNA under the *polI*⁺ promoter was sufficient to support growth of a *pcn1*⁺ disrupted strain and low enough to

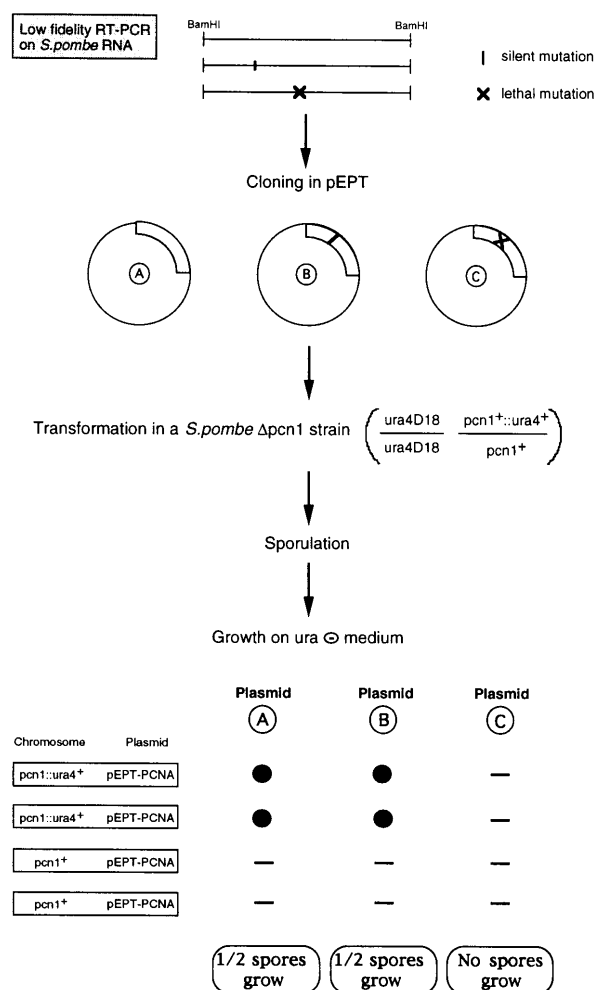


Figure 1. Strategy for identification of *S.pombe* PCNA lethal mutants.

avoid the toxic effect of PCNA (data not shown). The efficiency of mutagenesis was tested by sequencing a few isolated cDNAs resulting from the amplification. About four out of five clones were found to carry either one or two point mutations. Fifty independent pEPT/PCNA plasmids were each transformed into the diploid *S.pombe* strain disrupted for one allele of the *pcn1*⁺ gene and transformants were selected on appropriate medium. After selection of homothallic colonies by iodine staining, a defined number of spores were seeded on selective medium. The *pcn1*⁺ gene had been replaced by *ura4*⁺ in the disrupted strain, which is otherwise auxotrophic for this amino acid. Therefore, we looked for transformants from which half the spores could grow on uracil-containing medium (the *pcn1*⁺ spores) and no spores could grow on medium lacking uracil (the wild-type spores because they lacked *ura4*⁺ and the disrupted spores because they lacked a functional PCNA molecule). We thus identified eight clones unable to complement the disrupted strain. All viable spores were tested for cold (14°C) and heat (37°C) sensitivity, but no conditional mutants were identified. From the rate of mutation initially introduced by PCR that we evaluated, we would have expected to obtain a larger number of mutants. Although we have not sequenced the totality of the clones tested in our screen,

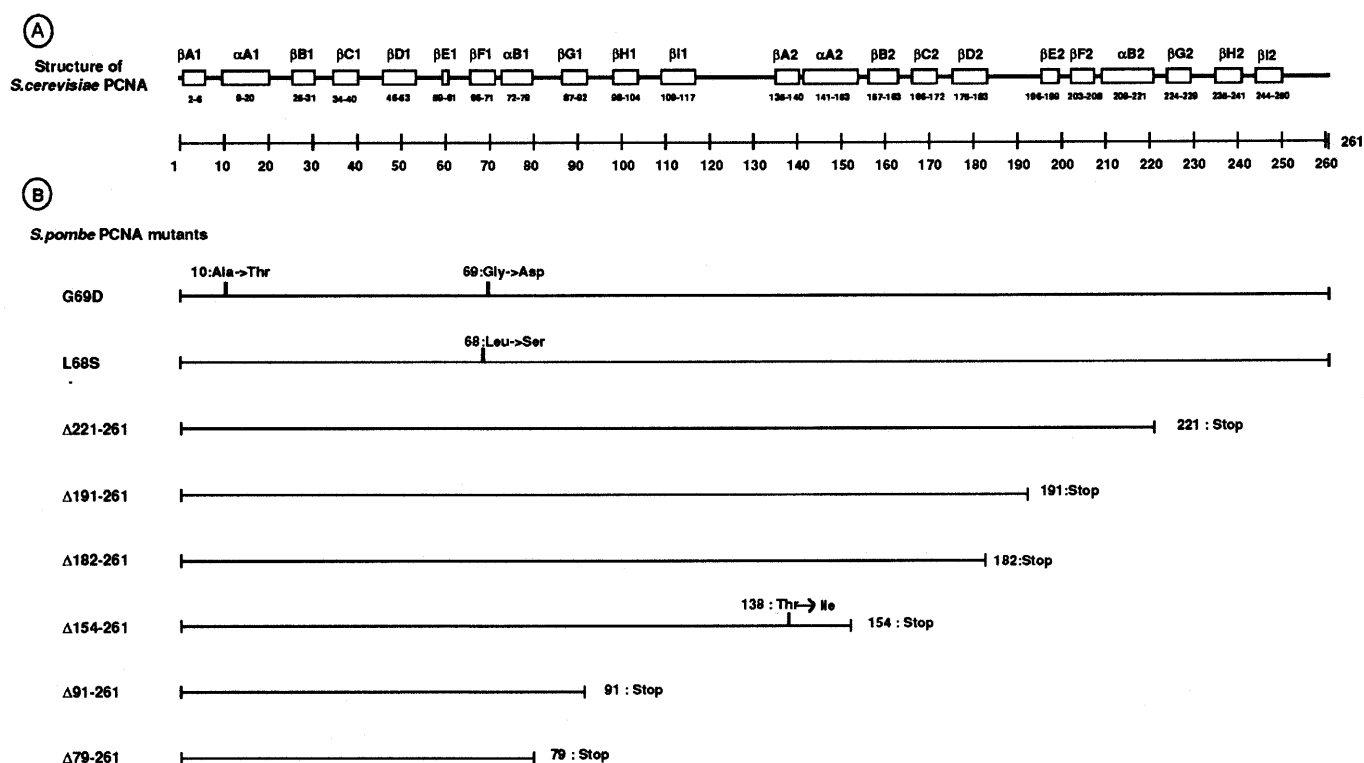


Figure 2. Localization of the *S.pombe* PCNA mutations on the *S.cerevisiae* PCNA structure. The *S.cerevisiae* PCNA structure is reproduced from Bauer and Burgers (19).

it is likely that a number of mutations remained silent. This would be in agreement with the previous observation that PCNA can withstand a large number of point mutations without undergoing adverse effects on its function (23,25).

Identification of the mutations

The cDNAs unable to complement the disrupted strain were sequenced to localize the positions of the mutations (Fig. 2). Two mutants exhibited point mutations that resulted in a single or double amino acid change. This did not affect the overall length of the protein. The double mutant was affected at positions 10 and 69 (AG10,69TD) and the single mutant was affected at position 68 (L68S). The two mutations of the double mutant were introduced separately on a *pcn1*⁺ cDNA and tested for their capacity to complement the disrupted *pcn1*⁺ strain. The A10T mutant was able to complement the disrupted strain, unlike the G69D mutant, which could not. Thus, the mutation of amino acid 10 was deemed to be silent and further analyses were performed on the single mutant G69D.

All the other mutants carried a mutation that generated a stop codon which resulted in deletions of various lengths of the C-terminus. The size of the deletion varied from 40 to 182 amino acids. It has been previously shown that a deletion as short as 11 amino acids of the C-terminus results in a non-functional human PCNA molecule (23). Therefore, it was not unexpected that none of these mutants were able to complement the disrupted strain. Because these mutants were likely to bear major structural

impairment, they were not further studied. Moreover, even the protein with the smallest deletion (Δ221–261) turned out to be largely insoluble when overexpressed in insect cells (data not shown).

To exclude the possibility that non-complementation was due to a lack of expression of the mutant PCNA protein from the plasmid introduced into the disrupted strain, total protein extracts were prepared from the diploid *S.pombe* disrupted strain transformed with a pEPT plasmid expressing wild-type PCNA or mutant L68S or G69D. The amount of protein detected in the cells transformed with a wild-type or a mutant plasmid was at least two times higher than that detected in the non-transformed disrupted strain, showing that exogenous protein had been produced (Fig. 3). Therefore, the lack of growth of the spores carrying a mutant plasmid cannot simply be due to a lack of expression of PCNA protein.

Purification of the mutant proteins expressed in insect cells

We have previously reported expression of *S.pombe* PCNA in insect cells using a recombinant baculovirus (29). The two mutants L68S and G69D cDNA were cloned into the pBACPAK1 vector to generate recombinant baculoviruses AcPcna68 and AcPcna69 respectively. Both mutant proteins were highly overexpressed in insect cells (see Fig. 4A, total extracts) but were slightly less soluble than the wild-type PCNA (data not shown). This suggests that the mutations of amino acids 68 and 69 affect the overall 3-dimensional structure of the protein. In the crude extracts of infected insect cells PCNA represented ~15% of the

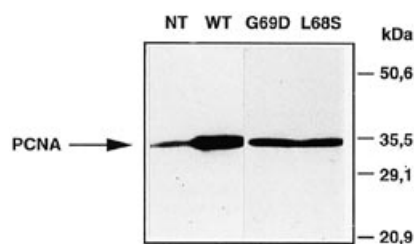


Figure 3. Expression of wild-type or mutant PCNA in *S.pombe*. The diploid *S.pombe* strain disrupted on one allele of the *pcn1*⁺ gene was transformed by either wild-type (WT) or mutant PCNA (G69D and L68S) expressed under the control of the *pol1*⁺ gene promoter. Aliquots of 100 µg total soluble proteins were separated on a 12% polyacrylamide gel and transferred onto nitrocellulose. PCNA proteins were detected with anti-PCNA antibodies (PC10). NT, non-transformed strain. Molecular size markers are indicated on the right.

total protein detected by Coomassie blue staining. After three steps of purification, a single band was detected by Coomassie blue staining (Fig. 4A). This band was recognized by anti-PCNA antibodies (Fig. 4B). In addition, these antibodies recognize a band of lower molecular weight whose intensity is correlated with the amount of PCNA detected (Fig. 4B). This is probably a degradation product of PCNA.

Functional characterization of the mutant proteins

The purified proteins were tested for their capacity to enhance the polymerase activity of *S.pombe* pol δ in an *in vitro* assay which measures the incorporation of [³H]dTTP using poly(dA)-oligo(dT) as template/primer. Both mutant proteins were unable to increase pol δ activity irrespective of the PCNA concentration (Fig. 5). In this assay, which employs a linear template and no RF-C, the PCNA trimer is loaded onto the DNA by simply sliding on from one of the extremities (35). So the main factors that could affect the capacity of PCNA to activate pol δ are its trimerization and/or its interaction with pol δ . Since PCNA is assembled as a trimer on DNA before recruiting pol δ , we first investigated the oligomeric status of the mutant proteins by cross-linking experiments. Cross-linking was performed with EGS, as previously described (34). At the optimal concentration of 2.5 mM EGS, the wild-type PCNA can be detected as a monomer, dimer and trimer (Fig. 6). We have no explanation for the presence of two bands at the dimer level. Maybe monomers can be cross-linked in two ways, leading to a differential migration of the dimer on denaturing gels. Increasing the incubation time of the reaction with wild-type PCNA resulted in an increase in the amount of trimer. At 300 s, almost no monomers were left in the reaction mixture. This is in agreement with previous reports showing that PCNA oligomers exist in solution as an equilibrium of these three forms (34). Under the same conditions, only dimers could be detected for both mutants. Dimer formation was more efficient for mutant L68S than for G69D, but was in both cases much less efficient than for the wild-type, as shown by the amount of monomers left after 300 s incubation. Therefore, the mutant PCNA are unable to form trimers at concentrations that allow the wild-type PCNA to adopt a trimeric structure. However, a significant number of dimers were detected, suggesting that monomers may still be able to interact together, but as an abnormal structure that is unable to

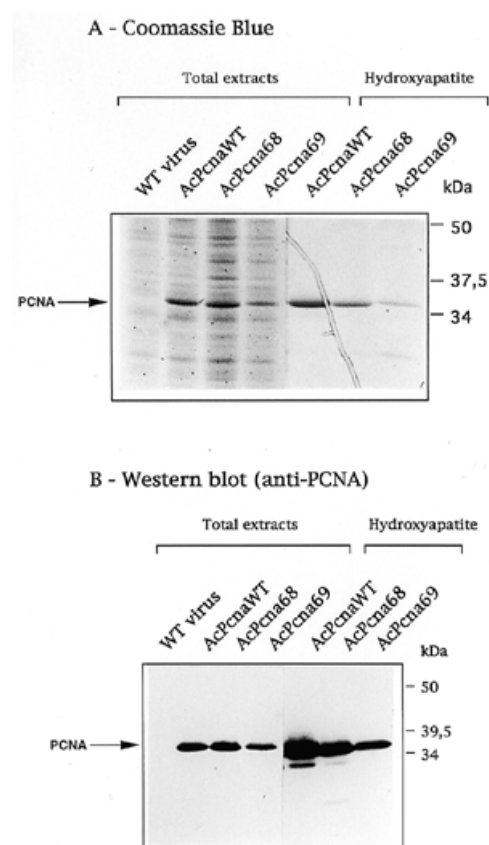


Figure 4. Overexpression of wild-type and mutant PCNA proteins in insect cells. Soluble proteins were extracted from Sf9 cells infected either with wild-type baculovirus or with wild-type or mutant PCNA recombinant viruses 48 h after infection. (A) Coomassie blue staining. Aliquots of 10 µg total soluble proteins (total extracts) or 2 µg purified protein (hydroxyapatite) were separated on 12% polyacrylamide gels and stained with Coomassie Blue. Molecular size markers are indicated on the right. (B) Western blot analysis. Aliquots of 2.5 µg total soluble proteins (total extracts) or 0.5 µg purified protein (hydroxyapatite) were separated on 12% polyacrylamide gels and transferred onto nitrocellulose. Recombinant proteins were detected with anti-PCNA antibodies. Molecular size markers are indicated on the right.

form a trimer. Increasing the mutant PCNA concentration in the cross-linking assay gave rise to a very small number of trimers (data not shown). These results suggest that the affinity of PCNA monomers for each other has been severely compromised by these mutations and that the lack of activation of pol δ by the PCNA mutants is due to their incapacity to form trimers.

It has been shown that polyethylene glycol (PEG) can restore the activity of a *S.cerevisiae* PCNA mutant unable to trimerize, by artificially increasing macromolecular crowding *in vitro* (25). We have investigated the effect of PEG on the ability of mutants L68S and G69D to activate pol δ *in vitro* (Fig. 7). Two concentrations of wild-type or mutant PCNA were tested in the presence or absence of 6% PEG. The wild-type PCNA activity was not affected by the presence of PEG and increased pol δ activity by the normal 10- to 15-fold. Our results show that the addition of PEG to the assay did not restore the activity of either mutant. In some instances a slight decrease in the affinity of monomers for each other can be compensated for by macromo-

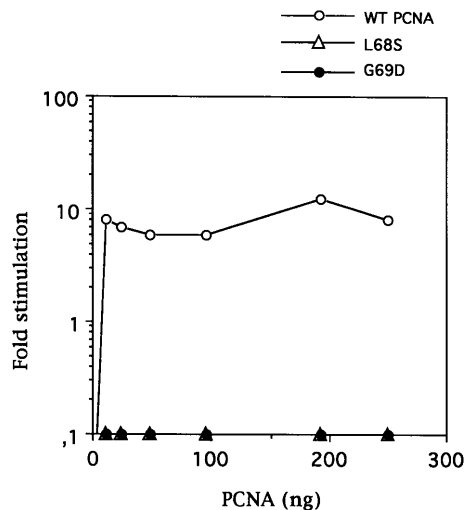


Figure 5. Stimulation of *S. pombe* DNA polymerase δ activity by wild-type and mutant *S. pombe* PCNA. The stimulation of *S. pombe* DNA polymerase δ was assayed by incorporation of [3 H]dTMP on poly(dA)-oligo(dT) in the presence or absence of increasing amounts of wild-type or mutant PCNA.

lecular crowding, which displaces the monomer/trimer equilibrium towards trimer formation. That this is not the case suggests that these PCNA mutants might have a defect that renders trimerization extremely unfavorable irrespective of the monomer concentration.

DISCUSSION

We have used a genetic screen to isolate lethal mutants of *S. pombe* PCNA. Among the 50 potential mutants tested, eight were identified that were unable to complement a *pcn1*⁺ disrupted strain. They all carried one or two single point mutations. Six mutants carried a mutation that introduced a stop codon in the coding sequence and this resulted in PCNA molecules that were truncated by a varying number of C-terminal amino acids. Two mutants were full-length PCNA that carried a mutation responsible for the lethal phenotype at amino acid 68 or 69. We overexpressed mutants L68S and G69D via recombinant baculoviruses, purified both proteins and characterized their biochemical defects. We showed that both proteins are unable to enhance the polymerase activity of pol δ due to their failure to form trimers. These results have several interesting implications. First, because of the high number of silent mutations that have been introduced into yeast and human PCNA, PCNA would appear to be able to withstand a large number of amino acid changes without any major functional alterations. Our results show that a single amino acid change may have dramatic effects on PCNA structure and function *in vivo*. A number of mutagenic studies have been performed on PCNA from different species. A systematic change of charged amino acids in human PCNA has identified amino acids essential for RF-C ATPase activity and pol δ activity *in vitro* (23). However, the effect of such mutations cannot easily be investigated *in vivo*. In the aforementioned study, only N- or C-terminal deletions of 8–11 amino acids resulted in major structural changes in the PCNA molecule, as indicated by their insolubility when overexpressed in *E. coli*. In *S. cerevisiae*, mutation of Ser115 to proline resulted in impairment of

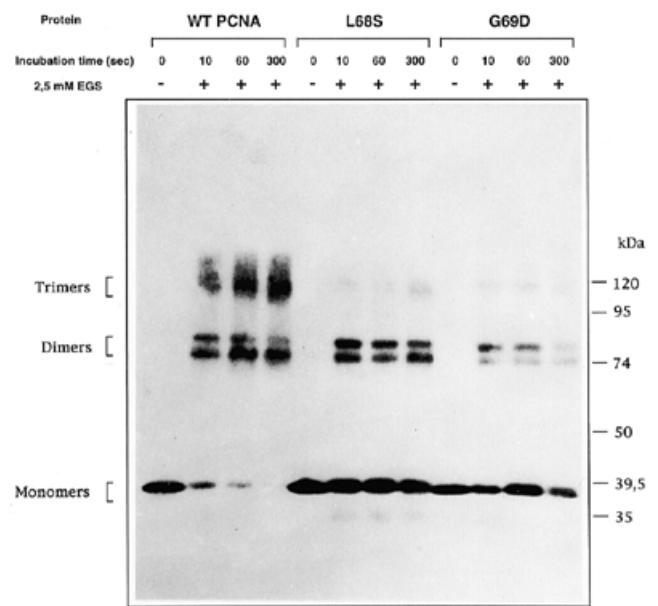


Figure 6. Cross-linking experiments with wild-type or mutant PCNA proteins. Purified wild-type or mutant PCNA was incubated for varying times, with or without EGS, at room temperature. The reaction products were separated on 8.5% polyacrylamide gels, transferred onto nitrocellulose and detected by anti-PCNA antibodies (PC10).

trimerization *in vitro* (25). Mutation of Tyr114 of human PCNA had a similar effect (24). Both amino acids 114 and 115 are located in the β -sheet β I1, which is within the inter-monomer region (20,21). Site-directed mutagenesis of *S. pombe* PCNA has identified residues whose mutation impaired stimulation of pol δ processivity (26). Most of these mutations could still support cell growth when expressed in a $\Delta pcn1$ strain. Interestingly, one of these mutations, unable to support growth of the disrupted strain, was a mutation of Gly69, but the mutant PCNA could not be fully characterized because of its insolubility when expressed in *E. coli*. By random mutagenesis and direct screening for lethal mutants, we have obtained the same mutation, further confirming the importance of this residue for PCNA structure. Expression in insect cells via a recombinant baculovirus allowed the production of a soluble protein and its purification. Obviously, the lack of solubility in *E. coli* suggests that the mutation must affect the structure of the protein significantly. However, in yeast itself and in higher eukaryotic cells, the mutant proteins were mainly recovered in the soluble fraction. It is well known that higher eukaryotic expression systems allow production of otherwise insoluble proteins, maybe because of the presence of chaperones that induce their proper folding. It may also be due to the lower levels of protein expressed over a longer time period.

Our screening has allowed identification of two adjacent mutations that result in a similar phenotype, i.e. loss of trimerization. This suggests that the region targeted by these two mutations must play a major structural role. We observed that the Gly69 mutation systematically resulted in a more pronounced phenotype than the Leu68 mutation. Mutant G69D was less soluble (not shown) and less efficient in dimer formation than L68S. Interestingly, Gly69 is conserved in all PCNA

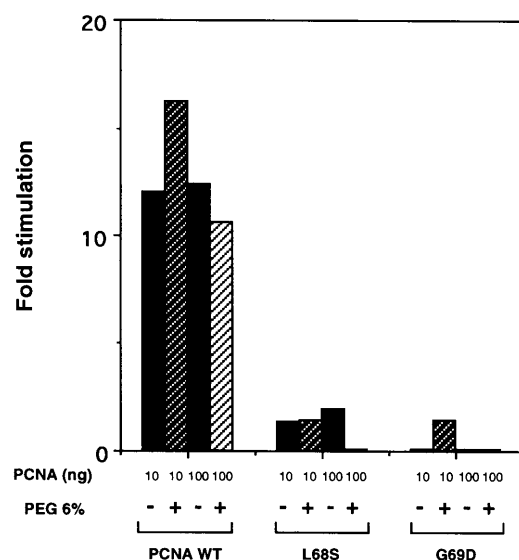


Figure 7. Effect of PEG on the activation of DNA polymerase δ by wild-type or mutant PCNA. Reactions were carried out as in Figure 5 with or without addition of PEG 8000 to a final concentration of 6%.

characterized so far, whereas Leu68 is not. From the *S.cerevisiae* PCNA 3-dimensional structure, amino acids 68 and 69 should be located in a β -sheet (β F1) not directly involved in monomer interactions. Although the 3-dimensional structure of *S.pombe* PCNA has not been determined, it is reasonable to expect that it will resemble that of human and *S.cerevisiae*, at least with respect to the distribution of α -helices and β -sheets, since it shares 52 and 44% sequence identity with these two species respectively. Therefore, this is the first report of a PCNA mutation affecting trimerization that is not located in the inter-monomer region. This observation suggests that the domain β F1 participates at a distance in maintaining the structure of the inter-monomer region, thereby allowing trimerization.

Although Gly69 is highly conserved in all species characterized so far, a similar mutation (Gly \rightarrow Asp) in *S.cerevisiae* has no phenotype by itself (36), which is a significant difference compared with the same mutation in *S.pombe*. However, it has been characterized as a suppressor of a cold-sensitive mutant of *CDC44*, the gene encoding the large subunit of RF-C. As previously mentioned, RF-C is the protein complex that loads PCNA onto DNA in an ATP-dependent manner. The current model proposes that RF-C opens the PCNA ring to allow PCNA loading on DNA. It has been hypothesized that *cdc44* mutants might be affected in ATPase activity. They would therefore be less efficient at loading PCNA onto DNA. Such mutation could be suppressed by an unstable PCNA trimer, which would then be loaded onto DNA at a lower energy cost. When resolving the *S.cerevisiae* PCNA crystal structure, Krishna *et al.* (20) observed that the C_{α} of Gly69 interacts with the side chains of amino acids 119 and 121 of the interdomain crossover loop. Because this loop has an important structural role in maintaining the integrity of the PCNA ring, they hypothesized that mutation of this residue had the potential to cause significant changes in the structure of PCNA and to affect the monomer-trimer equilibrium. Our results confirm this hypothesis and indicate that in *S.pombe*, this

mutation completely abolishes trimer formation both *in vivo* and *in vitro*. Apparently, in *S.cerevisiae*, this mutation has a less drastic effect and might only destabilize trimers. This would explain both the suppression of the *cdc44* mutation phenotype and the lack of a phenotype for this PCNA mutant by itself. Indeed, another *S.cerevisiae* PCNA mutant, *pol30-52* (25), which is unable to trimerize *in vitro* at any temperature, still supports cell growth at the permissive temperature, suggesting that trimer formation can be stabilized *in vivo* when PCNA is integrated into a replication complex.

Our results, together with those obtained by others, raise another important issue, i.e. one cannot simply rely on the conservation of primary structure to extrapolate the functional and structural roles of an amino acid residue. Obviously, Gly69 plays a major role in the structural cohesion of *S.pombe* PCNA, whereas it must have a less essential role in *S.cerevisiae* PCNA. Moreover, Leu68 is not conserved in all species where PCNA has been cloned. Similarly, mutation of a basic residue at position 146 [Arg in human (23), Lys in yeast (25)] abolished human PCNA stimulation of pol δ replication activity *in vitro* but had no effect on growth of *S.cerevisiae*. This latter observation could, however, be due to the previously mentioned discrepancy between the *in vitro* and *in vivo* results. Our screen overcomes that pitfall by directly identifying functional deficiencies *in vivo*. Comparison of the β subunit of *E.coli* DNA polymerase III and PCNA from *S.cerevisiae* has made it clear that very low primary sequence homology can still lead to a very high degree of conservation of the overall 3-dimensional structure, in this case the formation of a ring structure that can surround DNA. Still, the fine details of which residues precisely participate in the formation of the α -helices, β -sheets and interconnecting domain as well as interactions with other proteins cannot simply be deduced from superposition of the primary structures from each species. These observations suggest that the interactions that PCNA establishes with various factors of the replication and repair machinery, as well as its 3-dimensional structure, may need to be specifically defined in different species.

ACKNOWLEDGEMENTS

We thank S.Francesconi, D.Bouvier and M.Perderiset for providing the *pcn1* disrupted strain, the pEPT plasmid and the purified *S.pombe* pol δ respectively. We are grateful to A.Dias for excellent technical assistance and to P.Hughes and A.-M.De Recondo for critical reading of the manuscript. K.P. was the recipient of a fellowship from the Ministère de la Recherche et de l'Enseignement. This work was supported in part by grant 6704 from the Association pour la Recherche sur le Cancer and by European Union Contract no. FMRX-CT97-0125.

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